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STUDIES ON THE METHANOGENIC BACTERIA IN SLUDGE

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PROGRESS REPORT

STUDIES ON THE METHANOGENIC BACTERIA IN SLUDGE

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A summary of the progress of the project for the current period including discussion of procedural changes which have been instituted due to results obtained or problems encountered:

I. Fermentation studies

A. Intermediates

An objective of this project has been to quantitatively define the intermediate molecules preceding methane formation during sludge digestion. The assumption is made that if these intermediates are clearly defined the factors influencing their rates of metabolism can then be investigated, and that understanding of factors influencing these rates should suggest methods for controlling and increasing the rate of anaerobic digestion processes. The contribution of various intermediates as precursors of methane have been investigated using methods similar to those previously described by the principal investigator (1). Results are summarized in Table 1. Precursors determined during the grant period being summarized are shown with an asterisk. The data shows a quantitative evaluation of the major precursors of methane. It is likely that some methane is produced from hydrogen gas formed during the formation of the listed fatty acids but methods for quantitating the amount have not been successfully developed.

Our isotope turnover experiments have shown that the number two and three carbon of propionate are converted quantitatively to acetic acid and that the carbons of butyric and isobutyric acid can be accounted for on the basis of their conversion to acetic acid. During rapid digestion there is little or no exchange of labelled carbon between acetic acid and butyric acid or isobutyric acid, and labelled carbon dioxide is not incorporated into acetic acid.

The possible role of ethanol as a precursor of acetic acid has been investigated. The very rapid rate of disappearance of added ethanol in our sludge digester is shown in Figure 1. When ^{14}C labelled ethanol was added to the digesting sludge to a concentration of 7 μ moles per ml there was no significant change in specific activity of the ethanol during a period of 35 minutes, as shown in Table 2, although ethanol disappeared rapidly as would be anticipated from the data of Figure 1. Possible inhibitory effects of the high ethanol pool size were checked by decreasing the amount of ^{14}C labelled ethanol added. When ethanol was added to a concentration of approximately 1.0 μ moles per ml there appeared to be some turnover of the ethanol. The normal pool size of ethanol in our

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laboratory digesters is very small. In view of the slow turnover of ethanol the data indicates that ethanol does not contribute appreciably to the digestion process. Studies are now in progress to obtain a precise numerical value for the amount contributed.

Isovaleric acid was found to be present in small concentrations in the sludge of our digester. Turnover of added ^{14}C isovaleric acid was very slow. Turnover was determined by chromatographic methods to be described later in the report. A pool size of 0.35 μ moles per ml pool size of isovaleric acid remained constant during the course of the experiment. The counts in the isovaleric acid fractions are shown in Table 3. There was essentially no turnover of isovaleric acid and it is concluded that isovaleric acid is not a quantitatively significant contributor to methane evolution. It is recognized that this does not exclude the possibility of isovaleric acid being significant for the nutrition of the sludge microflora.

Efforts to evaluate a possible role of lactic acid in the digestion process have been initiated. Initial enzymatic determinations for lactic acid pool size gave values ranging from 0.15 μ moles per ml to 0.39 μ moles per ml.

Turnover data on lactic acid has not been determined because consistent results have not been obtained in our lactic acid analyses.

B. Methods

Methods have been previously described for the determination of the rates of various intermediate reactions during sludge digestion. These methods are limited in value, however, since they are very time consuming and require considerable manual dexterity. The possibility of evaluating the effects of inhibition or stimulatory substances by determining the effects of these substances on specific intermediate reactions was excluded due to the time which would be required for the chemical analysis. It was therfore decided to develop a method for rapid and accurate determination of the rates of intermediate reactions during the digestion process. This has been accomplished by combining a gas chromatograph, through a combustion furnace, to a flow cell in a scintillation counter.

Samples to be counted are injected into the gas chromatograph containing a column capable of separating the molecule of interest. The column effluent is split 16

to 1 with the major portion going to the flow cell. The minor portion is conducted to a flame ionization detector and response recorded on one channel of a dual-pen recorder. Radioactivity in the effluent is monitored by feeding the output of the scintillation counter into a rate meter and then into the second channel of the dual-pen recorder. Radioactive peaks are used to determine counting intervals. Actual counts in a specific fraction are printed out in digital form.

In order to check the linearity of the response of the gas chromatograph, the combustion furnace and the scintillation counter, a dilution experiment was conducted in which conditions were created similar to the events of acetic acid turnover in sludge. A solution was prepared containing approximately 1.0 μ c/ml ^{14}C acetic acid in a 10 μ mol/ml solution of acetic acid. The actual activity and concentration of acetic acid was determined and the solution diluted. Actual concentrations, counts, and specific activity were then calculated from the dilutions. The solutions were injected into the instrumentation previously described. If the system is functional for its designed purpose, calculated and measured values should be in close agreement. Results are shown in Table 4.

The close agreement between calculated and measured values clearly show that the method is applicable for the study of intermediate reactions during the digestion process. Development of this method now permits us to evaluate the effects of a wide variety of substances in the digestion process, since turnover values can now be quickly analyzed.

Turnover numbers having units of $min.^{-1}$ can be readily calculated from isotope dilution rates. The pool size of the particular intermediate in the sludge can be determined separately in units of μ moles per ml. The products of the two values show the extent to which a particular intermediate reaction is occurring in the sludge. At this time we are endeavoring to further simplify the system so that it will not be necessary to separately measure concentrations of the radioactive substance being studied. This is being attempted by incorporation of a radioactive, biologically inert, internal standard.

C. Inhibition Studies

In a previous progress report we concluded that the label in the two position of propionic acid can be recovered quantitatively in acetic acid if the determination is made over a short period of time, and that hydrogen gas specifically inhibits the metabolism of propionic acid in sludge.

An effort is now being made to determine the lowest concentration of hydrogen which will inhibit propionate metabolism, by measuring the inhibition of incorporation of ^{14}C labelled propionate into acetic acid. Concentration of hydrogen tested were, 4%, 9%, and 18%. Gases with these percentages of hydrogen, plus 30% CO_2 with a balance of nitrogen were equilibrated with digesting sludge and incorporation of ^{14}C labelled propionic acid into acetic acid measured with the results shown in Figure 2. Whether or not actual equilibration was obtained could not be determined due to the rapid uptake of the added hydrogen. The results show that a gas phase containing 9% hydrogen does not inhibit propionate metabolism but that propionate metabolism is inhibited by 18% hydrogen.

If the inhibition observed were due to oxidation-reduction changes in the sludge due to the added hydrogen it might be possible to reverse the inhibition by the addition of oxygen to the gas phase. Efforts to test this possibility have not been successful due to the fact that the concentrations of oxygen tested which influenced the system, also caused inhibition of the total fermentation. The experiments are continuing.

The effects of hydrogen on propionic acid metabolism in sludge, plus the large numbers of hydrogen utilizing methanogenic bacteria found in sludge, suggested investigation of the effect of hydrogen utilizing bacteria on the digestion process. Attempts to grow large masses of pure cultures of methanogenic bacteria in continuous culture have not been satisfactory for this purpose since the cell yield was low. In contrast it was possible to grow mixed cultures with good cell yields of hydrogen utilizing methanogenic bacteria, as indicated by rapid methane formation from hydrogen by the cell mass.

A 7 liter New Brunswick fermenter containing three liters of the 30 per cent rumen fluid medium used in culture work was inoculated with 150 ml of sludge supernatant and diffused with a mixture of 70% hydrogen and 30% carbon dioxide. The gas was recirculated using a diaphragm pump. Gas being pumped into the fermenter was passed through a hot copper column and an activated charcoal trap. The temperature of the fermentation was maintained at 35°C. After 72 hours the rate of methane evolution from the fermenter was 0.19 μ moles per ml per min., a rate approximately three times the rate of methane evolution from the digesters to be studied. One-half the cells could be harvested and fresh medium added with no decrease in total methane evolution after an additional 48 hours incubation. Cells in the two liters of fluid removed could be concentrated to a paste without loss of activity using an RC-2B centrifuge in tubes which had been made air free by gassing with a mixture of 70% nitro-

gen and 30% carbon dioxide.

Digester number 1 was fed raw sludge plus one-half of the cells harvested from the fermenter. The cells fed to digester 1 were viable. Digester number 2 was fed raw sludge plus the other one-half of the cells harvested from the fermenter. The cells fed to digester 2 were killed by steam sterilization prior to their use in the feed.

Cells were added periodically and the daily feed rate of raw sludge was increased slowly over a period of time, as shown in Figure 3.

The rate of gas evolution from the digesters was followed with time as shown in Figure 4. The propionic acid and acetic acid pools were followed with time as shown in Figures 5 and 6.

The results in Figure 4 show that the two digesters remained similar until the volume of raw sludge fed slightly exceeded 10% of the total volume of digesting sludge. The digester being fed viable methanogenic bacteria began to fail at that time. The control digester began to fail shortly after that. The slight difference in time at which the two digesters began to fail is not apparent. The results shown in Figure 5 indicate that addition of viable methanogenic bacteria resulted in a decrease in the propionic acid concentration in the sludge between the 30 and 38th day of the experiment. The results shown in Figure 6 indicate that the addition of viable methanogenic bacteria did not influence the acetic acid concentration in the sludge.

The results in Table 5 show that the bacteria added to the failing digester were capable of producing methane from H_2 and CO_2 but that after three days incubation in the failing digester the ability of the added cells to form methane from H_2 and CO_2 was only a seventh of the anticipated value. The results indicate the presence of some toxic substance which markedly decreases the activity of the added methanogenic bacteria. The nature of the inhibition is being studied. Our initial results indicate that the accumulation of acetic acid does not account for the inhibition. It is possible that in the absence of this inhibition a continued suppression of propionic acid accumulation could occur. This possibility is to be tested by altering the experimental design to include frequent addition of viable cells.

II. Pure culture studies.

A. Isolation studies

In the initial application it was pointed out that

few definitive data exist on the nature of the bacteria leading to methane formation in sludge and other environments. Kinds of methanogenic bacteria found in sludge have been recently described by the principal investigator (2). In these studies it was found that large numbers of different methanogenic bacteria could be isolated from sludge, all of which grew well on hydrogen gas as an oxidiable substrate. Utilization of other substrate was restricted to formic acid, acetic acid, and methanol. A total of six different species were described. Acetic acid was slowly metabolized by isolates having the characteristics of Methanosarcina barkeri. Continuation of these studies has resulted in the repeated isolation of these same six species.

Our isotope dilution studies previously described, have shown that approximately 95% of the total methane produced during digestion can be accounted for by the breakdown of acetic, propionic, n-butyric and isobutyric acid. It therefore seemed reasonable to anticipate that the pure culture isolates should be able to dissimilate these fatty acids. Repeated efforts under a wide variety of different cultural conditions failed to show any utilization of propionate or butyrate, and only slow utilization of acetate.

Enrichment cultures can readily be established from high dilutions of domestic sewage on all of the previously mentioned substrates. Enrichments were established using acetate, butyrate, or propionate as the only available energy sources. The enrichments were maintained for a period of several months prior to use in isolation studies. Samples from the enrichments were then diluted and methanogenic bacteria were isolated from dilutions of between 1×10^6 to 1×10^7. The same six species previously described were obtained from these dilutions. Studies on substrate utilization again showed no utilization of butyrate or propionate. Acetate was again slowly utilized. No correlation existed between the enrichment substrate used and the organism isolated. A number of possible explanations were examined.

Possibility one: The isolates possess the biochemical potential to metabolize fatty acids but do not express the potential in the environments of pure culture growth tested.

To test this possibility an effort was made to test the activity of the isolates in an environment closely simulating digesting sludge. Sludge with small amounts of fatty acids added was steam sterilized under an atmosphere of 70% N_2 and 30% CO_2, and then heavily inoculated with a methanogenic species. After one month of incuba-

tion the sludge was analyzed for the various fatty acids. Acids tested were acetate, propionate and butyrate. All six species were tested. Acetate disappeared in the sterile sludge inoculated with M. barkeri. The other tests showed no decrease in the concentration of acetate propionate or butyrate.

Since the results of this effort were negative an effort was made to create conditions more nearly simulating the digester environment than sterilized sludge. A non-metha e forming fermentation was established from digesting sludge. Such a fermentation was successfully created by a dilution procedure. One volume of digesting sludge was added to 9 volumes of steam sterilized sludge which had been actively fermenting. The mixture was incubated until a vigorous fermentation was obtained, as indicated by gas evolution. This occurred after approximately ten hours. The process was then repeated using the new fermentation as a source of inoculum. Gas evolved was analyzed for methane. After fifteen transfers the evolved gas contained no methane, even after long periods of incubation. Mixed fermentations obtained after 15 transfers were inoculated with the pure cultures, incubated for two weeks and analyzed for fatty acid disappearance. With the exception of acetate, as previously described, there was no disappearance of the fatty acids. The evidence obtained indicates that the isolates studied lack the capacity to utilize propionate or butyrate.

Possibility two: Hydrogen oxidizing, fatty acid dissimilating methanogenic bacteria exist but did not develop appreciably in our enrichments.

On the assumption that our enrichments procedures were not adequate, samples from an anaerobic filter were examined. Samples were kindly provided by Dr. Perry L. McCarty of Stanford University. Isolations of bacteria were made from a filter sample which had been fed for an extended period of time on a solution containing equal amounts of acetic and propionic acid as the sole carbon source. The samples were diluted and colonies isolated at random from the high dilutions using hydrogen as an energy source for growth. Fifty-five pure cultures were isolated of which 21 were methanogenic. All methanogenic species utilized hydrogen but would not utilize fatty acids, with the exception of several cultures of M. barkeri which utilized acetate. The methanogenic pure cultures obtained were similar to those previously mentioned, with some minor morphological variations.

Possibility three: Fatty acid dissimilating methanogenic bacteria exist but will not grow on hydrogen gas.

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Numerous efforts were made to isolate pure cultures using acetate, propionate, or butyrate as the sole energy source for growth. Anaerobic conditions were rigorously controlled. Gas phases used include 70% N_2, 30% CO_2, and 100% CO_2. The pH of the medium was appropriately buffered with $NaHCO_2$ and efforts were made at pH ranges between pH 6.5 and 7.5. Mixed cultures were obtained but all efforts to obtain pure cultures were without success.

On the assumption that these organisms might be cultivated on media containing a supplemental energy source efforts were made to isolate the fatty acid dissimilators using supplementary energy sources. Supplementary energy sources tested to this time include ethanol, pyruvate, glycerol, and acetaldehyde. All results have been negative.

On the assumption that the concentration of substrates might be critical for the growth of the desired organisms media were prepared with concentrations of sodium acetate of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1.0%, 1.5%, and 2.0% and inoculated with high dilutions of sludge. All isolation attempts were without success. The experiment was repeated using ammonium acetate. Pure cultures were not obtained. The experiment was not repeated for butyrate and propionate due to lack of time.

The effect of oxidation - reduction potential on the possible isolation of propionic acid dissimilators was checked. Medium was prepared containing added propionic acid but no added reducing agents. Resazurine was used as the oxidation - reduction indicator. The medium was heavily inoculated with E. coli and incubated for 72 hours, during which time the medium became reduced as shown by the color change of the resazurine. The E. coli was then killed by heat and the tubes inoculated with a heavy concentration of a methanogenic hydrogen utilizer. The tubes were incubated for three weeks and then the methanogenic organisms were heat killed. Various amounts of sterile water, containing dissolved O_2 were added to the culture tubes. The amount of water added was doubled for each succeeding set of tubes. Six sets of tubes were obtained before addition of oxygen to the tubes resulted in oxidation. The tubes were inoculated with dilutions from a propionic acid enrichment and incubated for three weeks. Tubes showing development of isolated colonies were examined for CH_4 production, and the water of syneresis was analyzed for propionate. CH_4 was not found and the propionate concentration did not differ from that of uninoculated controls. The oxidation reduction potential in the first tube with no added O_2 was approximately -360 mv as indicated by the reduction of reduced benzyl viologen.

Possibility four: Fatty acid dissimilating methanogenic

bacteria exist but will grow only in association with hydrogen utilizing methanogenic bacteria.

To test this hypothesis medium was prepared with added propionic acid and a gas phase of 100% CO_2. The media was then inoculated with a heavy suspension of Methanobacterium ruminantium. Enough M. ruminantium was added so that colony formation would not occur on incubation. The medium was further inoculated with dilutions of sludge in which a rapid metabolism of propionic acid was occurring. The concept was that if the propionic acid utilizer required symbiotic association with a hydrogen utilizing methane former, colonies of the propionic acid utilizer should develop and methane should be formed. The tubes were incubated for one month and analyzed for methane. None was produced. Examination of the tubes revealed the M. ruminantium had died during the course of the experiment, possibly due to the lack of an available energy source. Failure of this approach may have been due to death of the M. ruminantium. The experiment is to be repeated with modifications. M. ruminantium will be replaced by M. barkeri which remains viable for long periods of time in the absence of substrate. The experiments are being continued.

Possibility five: Fatty acid dissimilators exist which are not methanogenic but will metabolize fatty acids in conjunction with other metabolic activities.

None of our results are in disagreement with this possibility, the significance of which would be great to consideration of control of digestion and digestion rate. This is viewed by the principal investigator as one of the most important aspects of our current studies. The demonstration by Bryant (3) that M. omelianski was not a pure culture and that the ethanol oxidizing organism in the symbiotic system is not methanogenic, also supports this view. The work of Bryant (2) has been repeated in our laboratory with identical results.

An effort has been made to isolate organisms which will metabolize propionate without the formation of methane. Various media have been prepared with added propionate and inoculated with sludge and the metabolization of propionate followed. Rumen fluid media gave negative results. Organic media were tested. Sludge inoculation into medium containing mineral salts, 3% phytone, and 0.4% sodium propionate resulted in a 25% decrease in propionate concentration after 9 days incubation. One product had a retention time identical to propanol on gas chromatographic separation. To check this unexpected result the specific activity of ^{14}C labelled propionic acid was com-

pared to the specific activity of the propanol formed. The activities were identical. The possible relation of this observation to the digestion are being studied.

B. Culture collection.

All species of methanogenic bacteria which have been described as pure species exist in the culture collection with the exception of Methanococcus vannielii (4). Numerous efforts to isolate this organism by the principal investigator have been unsuccessful. Efforts by T.C. Stadtman to grow this organism from old stock and to reisolate this species have also been unsuccessful. Due to the unique structure and growth characteristics of this organisms efforts to reisolate it will continue.

Methods for culture maintenance, while successful, have been technically difficult. Methods for better preservation of these organisms were therefore studied. Earlier studies of this project showed that hydrogen peroxide formation was an important factor in the death of M. ruminantium when the organism was exposed to air. Since the reaction of pyruvate with peroxide is very rapid, the possible use of this substance as a protective and preserving agent against oxygen damage was examined. Medium was prepared containing 0.5% sodium pyruvate. Thirty % rumen fluid was included as an additional protective agent. This medium is the medium previously used to suspend cells for preservation in liquid nitrogen, except for the added pyruvate. The test organism selected was M. ruminantium. Tubes of media were divided into three sets, pH was controlled at pH 7 by appropriate adjustment with $NaHCO_3$.

Additives to each set are shown in Table 6. Each tube was inoculated with cells to a final cell population of approximately 1×10^8 cells per ml, and the tubes shaken for a period of 2 hours. Samples were then removed, quantitatively diluted, inoculated into the conventional medium, incubated and counted after two weeks incubation. Results are shown are shown in Table 7. The results show that 0.5 sodium pyruvate effectively protected the test organism against damage by exposure to air. This substance has therefore been incorporated into the suspending menstrum for the preservation of cultures. The following procedure has been developed for the preservation of the methanogenic bacteria.

1. A heavy cell suspension is suspended in 30% rumen fluid medium with added pyruvate.

2. A 0.5 ml sample of the suspension is injected into

a 2 ml glass vial which has been flushed air free with a mixture of 70% H_2 and 30% CO_2.

3. The glass vial is melted shut with a flame and the vial is placed on an aluminum retaining rack.

4. The vials containing cells are immersed directly into liquid nitrogen for storage.

5. As a standard procedure viability is checked after a short interval in the liquid nitrogen and again at intervals of approximately 6 months.

The procedure has been successful for all of the methanogenic bacteria.

Organisms included in the collection at this time include:

1. Methanobacterium formicicum

2. Methanobacterium ruminantium

3. Methanobacterium mobilis

4. The methanogenic member of the Methanobacterium omelianskii symbiotic.

5. The "Methanobacterium omelianskii" symbiotic mixture.

6. Methanosarcina barkeri

7. Four isolates which the principal investigator believes to be new species on the basis of morphological characteristics (These species will be described for the 8th edition of Bergy's Manual).

All cultures are available on request through the American Type Culture Collection.

C. Taxonomy

The principal investigator is a member of the Bergy's Manual Advisory Committee on Anaerobic Nonsporeforming Rods. He is specifically involved with the problem of the classification of the methanogenic bacteria. The problem of generic classification is straight forward. The genera of methanogenic bacteria validly described in the literature have been created on a morphological basis. The problem of species classification is, however, difficult since many of the organisms described in the literature have not been validly published. The descriptions do not conform to rule 14a in the International Code of

Nomenclature of Bacteria. In all cases the problem arises from the fact that the bacteria have been prematurely described, before pure cultures were obtained. Since the descriptions have been based on the properties of more than one species in mixed culture these species descriptions must be considered nomen rejiciendum and without standing in nomenclature. The invalid organisms include Methanobacterium propionicum, Methanobacterium sohngenii, Methanobacterium omelianskii, Methanobacterium suboxydans, Methanococcus mazei, and Methanosarcina methanica.

The recommendations being made for the classification of the methanogenic bacteria are summarized below.

Family - Methanobacteriaceae

Genus - Methanobacterium

1. M. formicicum
2. M. mobilus
3. M. ruminantium

Genus - Methanococcus

1. M. hydrogenophilus*
2. M. vannielii

Genus - Methanosarcina

1. M. barkeri

Genus - Methanospirillum

1. M. hungatei*

* These organisms will be described by the principal investigator for this classification. Two other species obtained in this investigation will not be described prior to completion of electron microscopy studies on their morphology.

References Cited

1. Smith, P.H. and R.A. Mah. 1965 Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. 14: 368-371.

2. Smith, P.H. 1966 The microbial ecology of sludge methanogenesis. Developments in Industrial Microbiol. 7: 156-161.

3. Bryant, M.P., et.al., 1967 Methanobacillus omelianskii, a symbiotic association of two species of bacteria. Archives fur Mikrobiologie. 59: 20-31.

4. Stadtman, T.C. and H.A. Barker. 1951 Studies on the methane fermentation. J. Bacteriol. 62: 269-280.

Table 1. Precursors of methane in domestic sludge

Precursor	% of total methane contributed ± 5%
Acetic acid	73
Propionic acid	13
n-butyric acid	8*
Isobutyric acid	1*
Total	95

Table 2. Turnover of ethanol added to sludge

Time in minutes after addition of ^{14}C ethanol	Specific activity picocuries per micromole
1	6.2
10	6.7
20	7.0
35	6.6

Table 3. Turnover of isovaleric acid added to sludge.

Time in minutes after addition of ^{14}C isovaleric acid	Counts per minute in isovaleric acid peak
0	499
5	501
75	483

Table 4. Test of gas chromatograph-scintillation counter system for turnover determination

Actual conc. of ^{14}C acetic acid µmoles/1ml	Mea. conc. of ^{14}C acetic acid µ moles/ml	Calc. counts in acetic acid peak	Mea. counts in acetic acid peak
10.2	10.2	1,999	1,999
5.10	5.15	1,000	1,038
2.55	2.49	500	482
1.28	1.32	250	254

Table 5. Effect of added methanogenic bacteria on hydrogen utilization and methane formation.

Time of cell Addition relative to experiment	Methane formation μ moles/ml/min. No H_2 added	Excess H_2 added	Increase due to added H_2
-3 days	0.005	0.013	0.008
0	0.005	0.103	0.098

Table 6. Additives to medium and tubes for study of protection of M. ruminantium from oxygen damage.

Set	Additive
1	Gas phase 70% H -30% CO_2, 0.05% Na_2S
2	Gas phase air, 0.5% sodium pyruvate
3	Gas phase air

Table 7. Effect of additives on viability of M. ruminantium following exposure to air.

	Total Colony Count		
Dilution	Set 1*	Set 2*	Set 3*
1 x 10	---	---	0
1 x 10	---	145	0
1 x 10	76	6	0

--- Colonies too numerous to count

* See table 6 for description

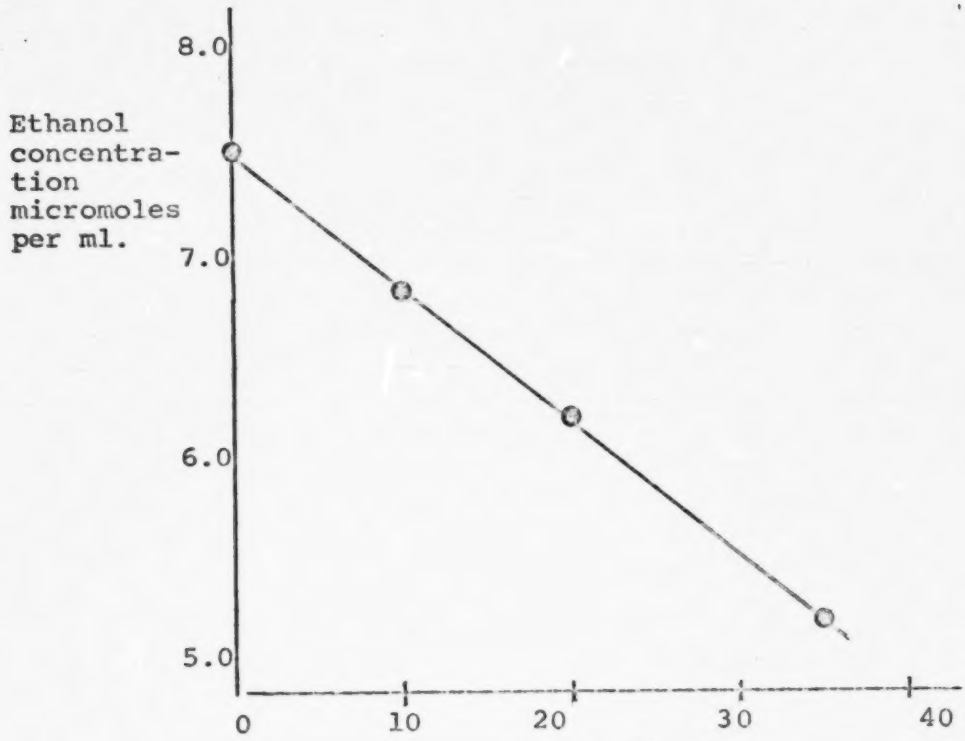

Figure 1. Disappearance of added ethanol from sludge.

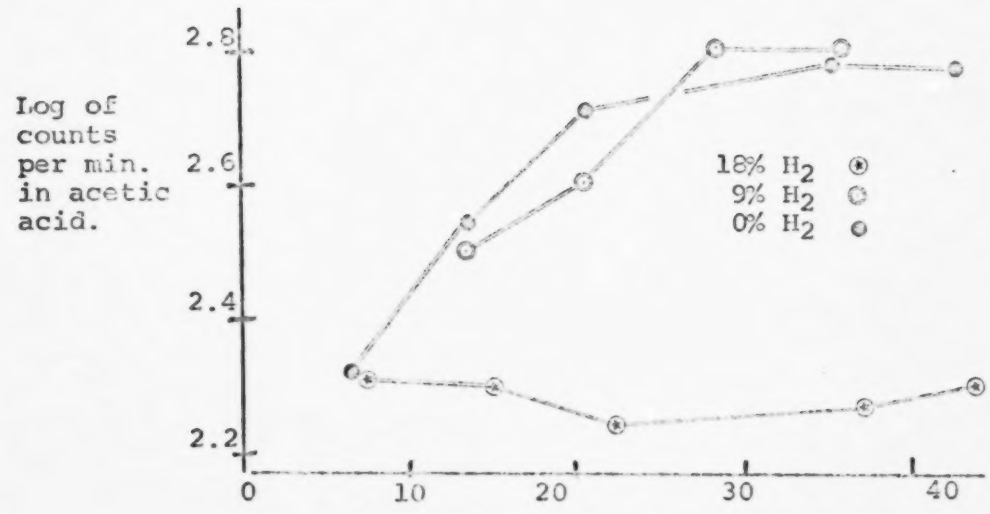

Figure 2. Effect of hydrogen on acetic acid production from propionic acid.

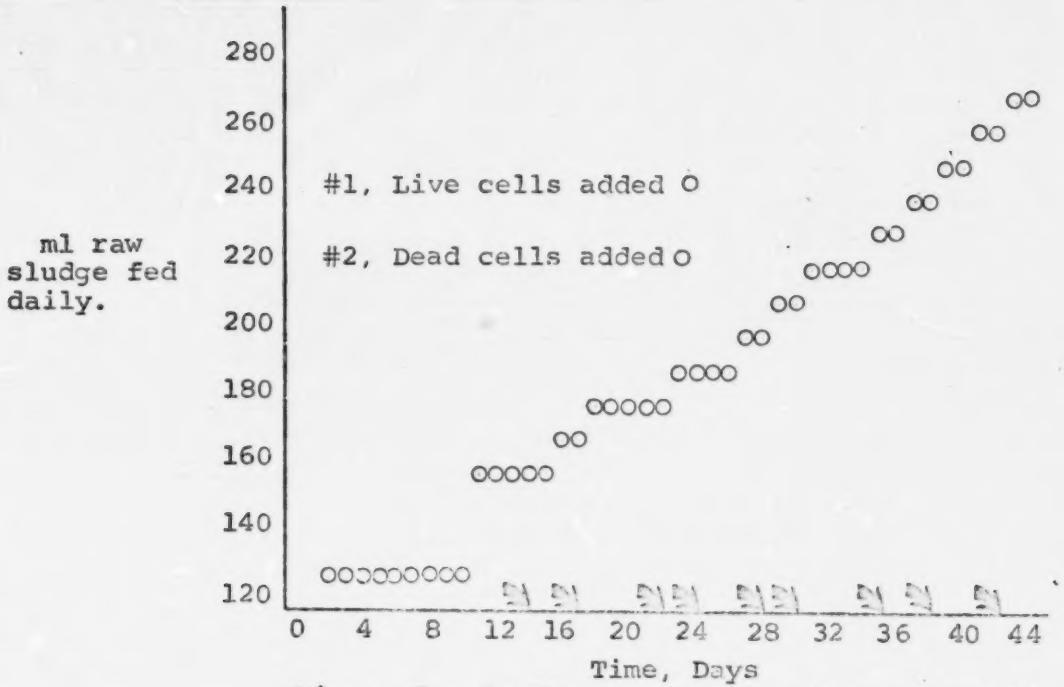

Figure 3. Feed rate for digesters #1 and #2.

(△)indicates days on which cells added.

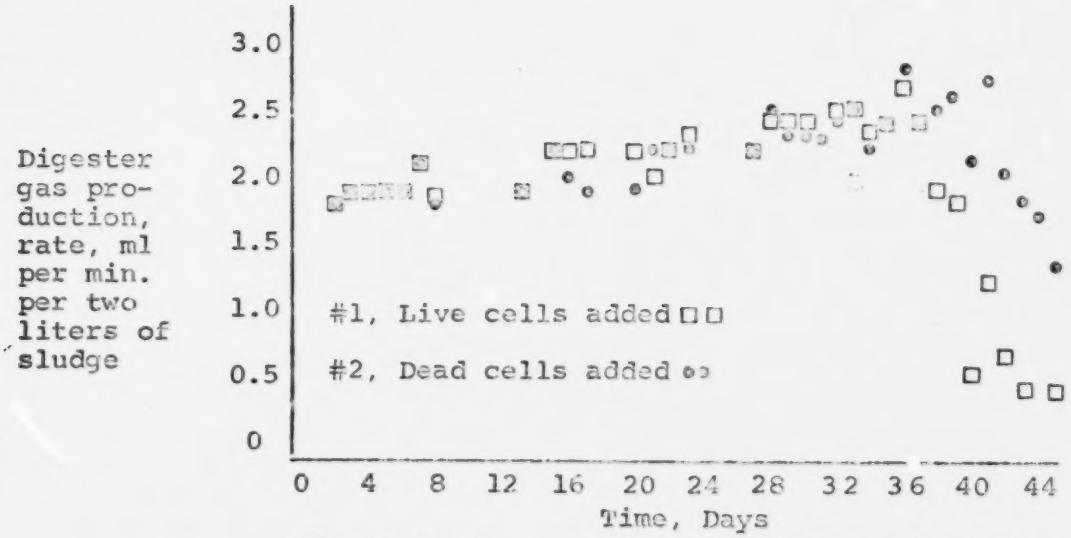

Figure 4. Gas production rates of digesters #1 and #2 during first four hours after feeding.

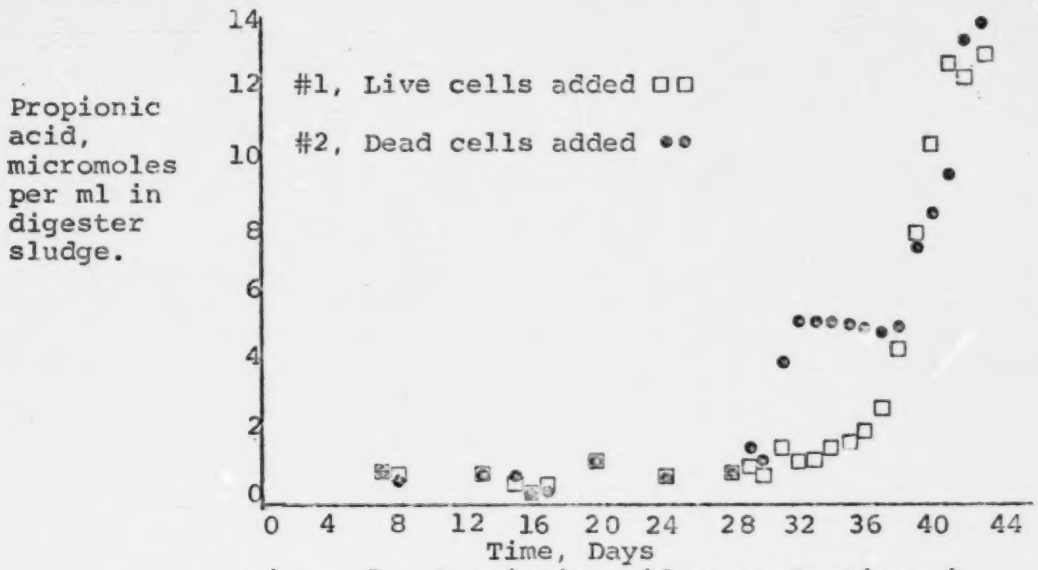

Figure 5. Propionic acid concentrations in digesters #1 and #2, four hours after feeding.

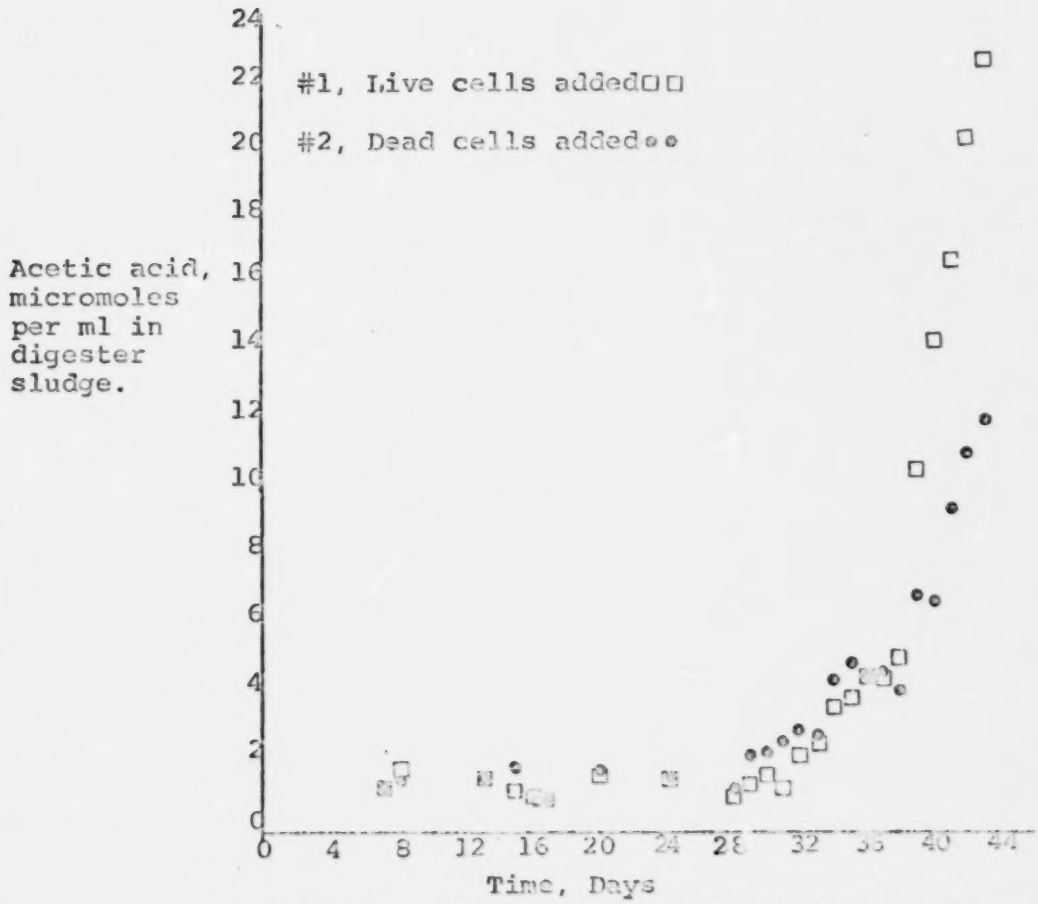

Figure 6. Acetic acid concentrations in digesters #1 and #2, four hours after feeding.